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Follicular Dendritic Cell Dedifferentiation by Treatment with an Inhibitor of the Lymphotoxin Pathway Dramatically Reduces Scrapie Susceptibility

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Transmissible spongiform encephalopathies (TSEs) may be acquired peripherally, in which case infectivity usually accumulates in lymphoid tissues before dissemination to the nervous system. Studies of mouse scrapie models have shown that mature follicular dendritic cells (FDCs), expressing the host prion protein (PrP^c), are critical for replication of infection in lymphoid tissues and subsequent neuroinvasion. Since FDCs require lymphotoxin signals from B lymphocytes to maintain their differentiated state, blockade of this stimulation with a lymphotoxin β receptor-immunoglobulin fusion protein (LTβR-Ig) leads to their temporary dedifferentiation. Here, a single treatment with LTβR-Ig before intraperitoneal scrapie inoculation blocked the early accumulation of infectivity and disease-specific PrP (PrP^{Sc}) within the spleen and substantially reduced disease susceptibility. These effects coincided with an absence of FDCs in the spleen for ca. 28 days after treatment. Although the period of FDC dedifferentiation was extended to at least 49 days by consecutive LTβR-Ig treatments, this had little added protective benefit after injection with a moderate dose of scrapie. We also demonstrate that mature FDCs are critical for the transmission of scrapie from the gastrointestinal tract. Treatment with LTβR-Ig before oral scrapie inoculation blocked PrP^{Sc} accumulation in Peyer's patches and mesenteric lymph nodes and prevented neuroinvasion. However, treatment 14 days after oral inoculation did not affect survival time or susceptibility, suggesting that infectivity may have already spread to the peripheral nervous system. Although manipulation of FDCs may offer a potential approach for early intervention in peripherally acquired TSEs, these data suggest that the duration of the treatment window may vary widely depending on the route of exposure.

The transmissible spongiform encephalopathies (TSEs) or "prion diseases" are chronic neurodegenerative diseases that affect humans and both wild and domestic animals. Most TSEs, including natural sheep scrapie, bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD) in mule deer and elk, and variant Creutzfeldt-Jakob disease (vCJD) in humans, are most likely transmitted by peripheral exposure. For example, the emergence of vCJD in the United Kingdom population is almost certainly due to consumption of BSE-contaminated meat products (7, 25). After peripheral inoculation with TSE agents, high levels of infectivity and the disease-specific isoform of the host prion protein (5) (PrP^{Sc}) usually accumulate in lymphoid tissues prior to their detection in the central nervous system (CNS). This accumulation in lymphoid tissues appears critical for efficient neuroinvasion (19), suggesting an important role for the immune system in the propagation of infectivity from the periphery to the CNS.

Within the lymphoid tissues of patients with vCJD (24), most sheep with natural scrapie (56), or after experimental peripheral inoculation of rodents with scrapie (6, 28, 37, 39, 44), early PrP^{Sc} accumulation takes place in the germinal centre on follicular dendritic cells (FDCs). Studies of mouse scrapie models have shown that mature FDCs are critical for replication in lymphoid tissues and, in their absence, neuroinvasion after peripheral challenge is significantly impaired (6, 17, 31, 37–39, 46, 51). From the lymphoid tissues infectivity spreads to the CNS via peripheral nerves (21).

Cytokines produced by B lymphocytes, such as lymphotoxins (LTs), provide essential signals for FDC development and maturation (10, 34). Studies from genetically immunodeficient mice have shown that in the absence of LTα (43) or LTβ (33) the development of FDC networks is blocked. The membrane-bound form of LT ($LT\alpha_1\beta_2$ heterotrimer) is expressed on activated lymphocytes, and signaling is mediated through the LTβ receptor (LTβR) expressed on stromal cells such as FDCs (12). Signaling via the LTβR is likewise necessary for the development of FDCs as they do not develop in mice deficient in this receptor (20). In adult mice, the maintenance of preexisting FDC networks in a differentiated state also requires continual stimulation through the LTβR since they rapidly collapse from their mature state when signaling is blocked by a specific inhibitor of the LTβR pathway (40).

We and others have previously shown that, in the temporary absence of mature FDCs after treatment with LTβ receptor-immunoglobulin fusion protein (LTβR-Ig [16]), early scrapie accumulation in the spleen is blocked and neuroinvasion is significantly delayed (37, 46). Since these studies imply that FDCs could be targeted for early therapeutic intervention against TSE agents, further experiments were performed in the current study to address the following questions. First, does treatment with LTβR-Ig reduce susceptibility to peripherally inoculated scrapie and, if so, to what extent? Second, since the effects of LTβR-Ig on FDC status are temporary (22, 40), do

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consecutive doses of LTβR-Ig prolong the period of FDC dedifferentiation and, if so, does this further reduce disease susceptibility? Finally, since early PrP^{Sc} accumulation occurs upon FDCs after oral inoculation of rodents (3) and sheep (1, 23) with scrapie and of mule deer with CWD (54), are FDCs important for TSE pathogenesis after oral inoculation? To address this question, mice were treated with LTβR-Ig before or shortly after oral scrapie inoculation to determine whether temporary FDC dedifferentiation reduced susceptibility to orally inoculated scrapie and, if so, how long after inoculation such intervention was possible?

MATERIALS AND METHODS

LTβR-Ig treatment. C57BL mice (8 to 12 weeks old) were given a single intraperitoneal (i.p.) injection of a fusion protein containing the soluble LTβR domain linked to the Fc portion of human immunoglobulin G1 (IgG1; LTβR-Ig [16]) or 100 μg of polyclonal human IgG (hu-Ig; Sandoglobulin) as a control. In some cases, mice were given one or two subsequent doses of 100 μg of LTβR-Ig or 100 μg of hu-Ig at 21-day intervals.

Scrapie infection. At the times indicated relative to treatment, mice were injected i.p. with 20 μl of a 1.0, 0.1, 0.01, or 0.001% (wt/vol) dilution of unspun brain homogenate from C57BL mice terminally affected with ME7 scrapie (20 μl of a 1.0% homogenate represents a dose of ca. $10^{4.5}$ intracerebral [i.c.] ID_{50} [50% infectious dose] units). For oral inoculation, mice were fed individual food pellets doused with 50 μl of a 1.0% scrapie brain homogenate. After challenge, animals were coded and evaluated weekly for signs of clinical disease and then killed at a standard clinical endpoint (18). Scrapie diagnosis was confirmed by histopathological assessment of vacuolation in the brain. Where indicated, some mice were sacrificed 70 days postchallenge, and spleens, mesenteric lymph nodes (MLNs), and Peyer's patches were obtained for further analysis. For bioassay of scrapie infectivity, individual half-spleens were prepared as 10% (wt/vol) homogenates in physiological saline, and 20-μl portions were injected i.c. into groups of 12 C57BL indicator mice. The scrapie titer in each spleen was determined from the mean incubation period in the assay mice by reference to established dose- and incubation period-response curves for scrapie-infected spleen tissue (11).

Immunohistochemical analysis. To monitor the effects of treatment on FDC status tissues were taken from two mice from each group at the times indicated after treatment and snap-frozen at the temperature of liquid nitrogen. Frozen sections (6 μm) were cut on a cryostat, and FDCs visualized by staining with FDC-specific rat monoclonal antiserum FDC-M1 (BD Pharmingen, San Diego, Calif.) or FDC-M2 (AMS Biotechnology, Abingdon, United Kingdom) or 8C12 monoclonal antiserum to detect CD35 (BD Pharmingen). Complement components were detected by using rat monoclonal antiserum RMC7H8 specific for C1q (Connex, Martinsried, Germany) or RMC11H9 specific for C3 (Connex). Immunolabeling was carried out by using alkaline phosphatase coupled to the avidin-biotin complex (Vector Laboratories, Burlingame, Calif.). Vector Red (Vector Laboratories) was used as a substrate.

For the detection of PrP in brain tissue and Peyer's patches, tissues were fixed in periodate-lysine-paraformaldehyde and embedded in paraffin wax. Sections (6 μm) were deparaffinized and pretreated to enhance PrP immunostaining by hydrated autoclaving (15 min, 121°C, hydration) and subsequent immersion in formic acid (98%) for 5 min (44). Sections were then stained with the PrP-specific polyclonal antiserum 1B3 (14) and immunolabeling was detected by using hydrogen peroxidase coupled to the avidin-biotin complex (Vector Laboratories) with diaminobenzidine as a substrate. Glial fibrillary acid protein (GFAP) was detected on adjacent brain sections by using rabbit GFAP-specific antiserum (Dako, Ltd., Ely, United Kingdom), and immunolabeling was carried out by using alkaline phosphatase coupled to the avidin-biotin complex (Vector Laboratories) with Vector Red as a substrate.

All sections were counterstained with hematoxylin to distinguish cell nuclei.

Immunoblot detection of PrP^{Sc}. Spleen fragments or MLNs (approximately half the total from each mouse assayed) were prepared as previously described (13, 36, 39). Briefly, before immunoblot analysis, tissue homogenates were treated with 40 μg of proteinase K (to confirm the presence of PrP^{Sc}) and subsequently partially purified by treatment with 2% (wt/vol) *N*-lauroylsarcosine (in 0.1 M Tris [pH 7.4]), allowing sedimentation only of the proteinase K-resistant, detergent-insoluble fraction of PrP (PrP^{Sc}). Samples were subjected to electrophoresis through sodium dodecyl sulfate–10 to 20% polyacrylamide gels (Bio-Rad, Hemel Hempstead, United Kingdom) and then transferred to polyvinylidine difluoride membranes (Bio-Rad) by semidry blotting. PrP was detected with the PrP-specific mouse monoclonal antiserum 8H4 (59), followed by alkaline phosphatase-conjugated goat-anti mouse antiserum (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.), and bound alkaline phosphatase activity was detected with SigmaFast NBT/BCIP solution (Sigma, Poole, Dorset, United Kingdom).

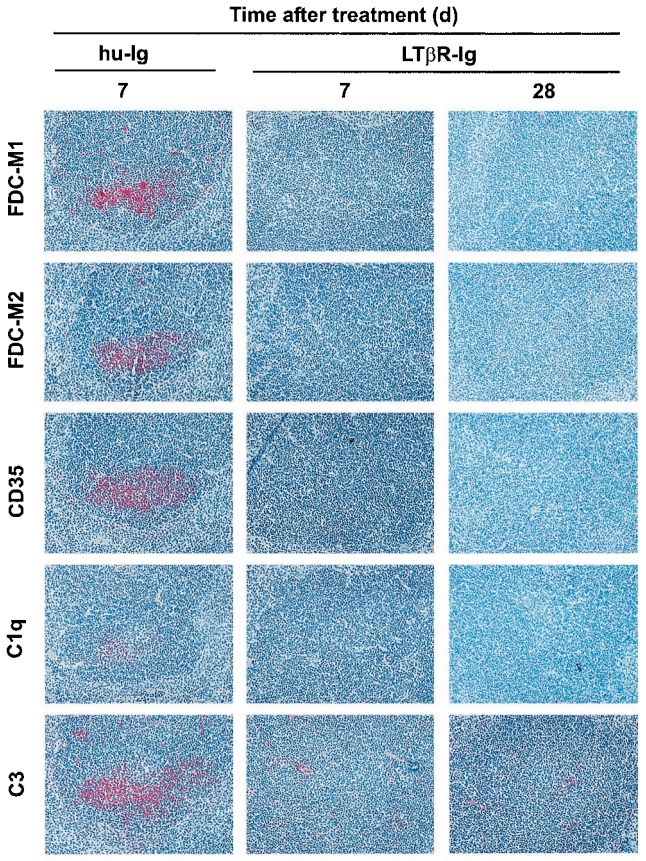

FIG. 1. Effect of LTβR-Ig treatment on FDC status in spleens of uninfected mice. Tissues were taken on the days indicated postinjection with hu-Ig (control) or LTβR-Ig, and adjacent frozen sections stained with FDC-M1 (top row; red) and FDC-M2 (second row; red) monoclonal antiserum to detect FDCs, 8C12 monoclonal antiserum to detect CD35 (third row; red), and monoclonal antiserum specific for complement components C1q (fourth row; red) and C3 (lower row; red). Expression of FDC-M1, FDC-M2, CD35, and associated accumulations of complement components C1q and C3 were undetectable in the spleen after treatment with LTβR-Ig. Original magnification, ×400.

RESULTS

Effect of LTβR-Ig treatment on FDC status. Temporary blockade of the LTβR signaling pathway was achieved by a single i.p. injection of 100 μg of LTβR-Ig (16). After treatment of mice with LTβR-Ig, expression of the FDC markers FDC-M1, FDC-M2, and CD35 (complement receptor 1) was undetectable in the spleen 3 days (data not shown) and 7 days (Fig. 1) after treatment. FDCs characteristically trap and retain antigens on their surfaces through interactions between complement components and cellular complement receptors (47, 50). Here, the loss of expression of complement receptor 1 (Fig. 1) in the spleens of LTβR-Ig-treated mice coincided with a sub-

TABLE 1. Effect of LTβR-Ig treatment on susceptibility to i.p.-inoculated scrapie[a]

% Inoculum dilution (wt/vol)	hu-Ig		LTβR-Ig	
	Incidence	Mean incubation period (days) ± SE	Incidence	Mean incubation period (days) ± SE
1.0	8/8	296 ± 8	5/7	341 ± 22, 2× > 500
0.1	8/8	337 ± 5	2/8	322, 354, 6× > 550[b]
0.01	7/9	330 ± 8, 2× > 550	0/7	7× > 550[c]
0.001	0/8	8× > 550	0/8	8× > 550[d]

[a] Incidence = number of animals affected/number of animals tested. The notation "$N \times > 500$" means that mice were free of the signs of scrapie up to at least this time after inoculation.

[b] One mouse was killed 541 days after inoculation. No histopathological signs of scrapie were detected in the brain (data not shown).

[c] One mouse was killed 386 days after inoculation. No histopathological signs of scrapie were detected in the brain (data not shown).

[d] Three mice were killed 326, 371, and 515 days after inoculation. No histopathological signs of scrapie were detected in their brains (data not shown).

stantially decreased abundance of complement components C1q and C3 (Fig. 1). The remaining complement-specific immunostaining is likely to represent macrophage-associated C1q and C3 (15, 52). Taken together, these data imply that after treatment with LTβR-Ig the FDCs, if present, were dedifferentiated and had lost their ability to trap and retain antigens in the germinal center. Although the effects of LTβR-Ig treatment on FDC status are temporary, little evidence of recovered FDC networks was detected in the spleen 28 days after treatment (Fig. 1). Treatment of mice with 100 μg of polyclonal human IgG (hu-Ig) as a control had no adverse effect on FDC status 7 days (Fig. 1) or 28 days (data not shown) after injection.

Effect of LTβR-Ig treatment on scrapie susceptibility. Mice were given a single i.p. injection of LTβR-Ig (or hu-Ig as a control) 3 days before i.p. inoculation with a moderate or limiting dose of scrapie. Treatment with LTβR-Ig prior to scrapie inoculation significantly reduced disease susceptibility and extended survival time (Table 1). For example, after injection with a moderate dose of scrapie (20 μl of a 1.0% scrapie brain homogenate), all control-treated mice succumbed to disease with a mean incubation period of 296 ± 8 days ($n = 8$), whereas five of seven mice treated with LTβR-Ig developed disease 45 days later, with a mean incubation period of 341 ±22 days (Table 1). Two LTβR-Ig-treated mice remained free of signs of scrapie 500 days postinoculation. Characteristic spongiform pathology, PrP^{Sc} accumulation, and reactive astrocytes expressing high levels of GFAP were detected in the brains of all LTβR-Ig-treated and hu-Ig-treated control animals which developed clinical signs of scrapie (data not shown). In contrast, spongiform pathology, PrP^{Sc} accumulation or reactive astrocytes were not detected in the brains of the two surviving LTβR-Ig-treated mice (data not shown). When mice were inoculated with scrapie directly into the CNS by intracerebral injection, treatment with LTβR-Ig prior to scrapie challenge had no effect on the incubation period of disease or pathology within the brain compared to controls (37; data not shown). This finding is consistent with the lack of transfer of high-molecular-weight soluble receptors and IgG through the blood-brain barrier (49).

Treatment of mice with LTβR-Ig prior to i.p. inoculation with lower doses of scrapie (20 μl of a 0.1% or 0.01% scrapie brain homogenate) demonstrated a striking effect on disease susceptibility (Table 1). When mice were treated with hu-Ig 3 days before injection with a 0.1% scrapie brain homogenate, all mice developed neurological disease with a mean incubation period of 337 ± 5 days ($n = 8$; Table 1). In contrast, six of eight mice treated with LTβR-Ig remained free of the signs of scrapie for at least 550 days after inoculation. Comparisons of disease susceptibilities between mice treated with LTβR-Ig and hu-Ig suggest that blockade of the LTβR-signaling pathway reduced disease susceptibility approximately 100-fold.

Scrapie infectivity and PrP^{Sc} accumulation in the spleen. Within 70 days of an i.p. injection of untreated mice with the ME7 scrapie strain, high levels of infectivity and the disease-specific isomer of the prion protein, PrP^{Sc}, accumulate within lymphoid tissues (6, 13, 36, 39). In the present study, spleens were taken from two control and two LTβR-Ig-treated mice 70 days after i.p. injection with a moderate dose of scrapie (1.0% scrapie brain homogenate) and halved. PrP^{Sc} accumulation was determined in one half by immunoblot analysis, whereas the scrapie infectivity titer in the other half was estimated by bioassay in groups of 12 indicator mice. As expected, spleens from control mice treated with hu-Ig 3 days before scrapie challenge contained high levels of infectivity (ca. 5.7 log i.c. ID_{50}/g as estimated by incubation period assay; Fig. 2a) and abundant detergent-insoluble, relatively proteinase-K-resistant PrP^{Sc} (Fig. 2a, lanes 2 and 4). However, after treatment of mice with LTβR-Ig 3 days before scrapie challenge, no PrP^{Sc} and only trace levels of infectivity were detected in the spleen 70 days postinoculation (Fig. 2a, lanes 6 and 8). This finding is consistent with long-term persistence of a fraction of the infectivity from the inoculum (17).

High levels of PrP^{Sc} accumulation were also detected at the terminal stage of disease in the spleens of all hu-Ig-treated control animals that developed clinical signs of scrapie (Fig. 2b, lanes 2 and 4). The effects of LTβR-Ig treatment on FDC status are temporary, and mature networks begin to reappear approximately 28 days after injection. Thus, the detection of high levels of PrP^{Sc} in the spleen at the terminal stage of disease of all LTβR-Ig-treated animals that developed clinical signs of scrapie is consistent with replication on the recovered FDC networks (Fig. 2b, lanes 10 and 12). In contrast, PrP^{Sc} was undetectable in the spleen of one mouse that remained free of the signs of scrapie 500 days after inoculation, implying that this mouse would have been unlikely to develop clinical disease (Fig. 2b, lane 8). However, high levels of PrP^{Sc} were detected in the spleen of the other mouse that was free of the signs of scrapie 500 days after inoculation (Fig. 2b, lane 6). Although characteristic signs of neurodegeneration and PrP^{Sc} accumulation were not detected in the brain 500 days after inoculation (data not shown), this mouse may have developed clinical scrapie after a substantially prolonged incubation period.

Effect of multiple LTβR-Ig treatment on scrapie susceptibility. Since the effects of LTβR-Ig treatment on FDC status are temporary, we next sought to determine whether prolonged FDC dedifferentiation would further reduce susceptibility to inoculation with a moderate dose of scrapie. To achieve this, mice were given a single i.p. injection of 100 μg of LTβR-Ig (or 100 μg of hu-Ig as a control) 3 days before i.p. inoculation with a 1.0% scrapie brain homogenate. After

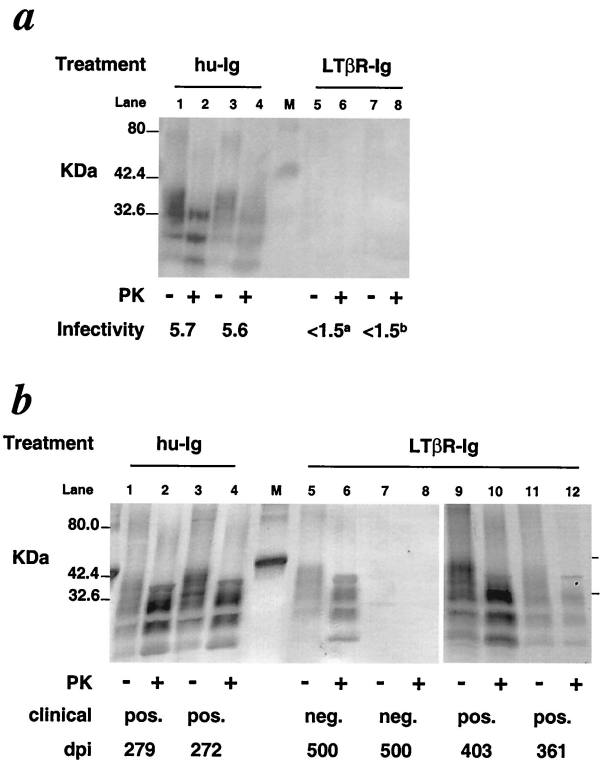

FIG. 2. Treatment with LTβR-Ig prior to i.p. scrapie inoculation blocks the early accumulation of PrP^{Sc} and infectivity in the spleen. Mice were treated with LTβR-Ig or hu-Ig (as a control) 3 days before i.p. scrapie inoculation, and tissues were assayed 70 days after inoculation (a) or at the terminal stage of disease (b). Immunoblots show the accumulation of detergent insoluble, relatively proteinase K (PK)-resistant PrP^{Sc}. Treatment of tissue in the presence (+) or absence (−) of proteinase K before electrophoresis is indicated. After proteinase K treatment, a typical three-band pattern was observed between molecular mass values of 20 and 30 kDa, representing unglycosylated, monoglycosylated, and diglycosylated isomers of PrP (in order of increasing molecular mass). PrP was detected by using the PrP-specific monoclonal antiserum 8H4. Lane M contained molecular mass markers. The scrapie infectivity titer is expressed as the log i.c. ID_{50} units/g. (a) A total of 1 of 12 assay mice developed clinical scrapie within 500 days of inoculation; (b) 2 of 12 assay mice developed clinical scrapie within 500 days of inoculation. dpi, day postinoculation on which the tissues were analyzed; pos., mice that developed clinical signs of scrapie; neg., mice that were free of the clinical signs of scrapie.

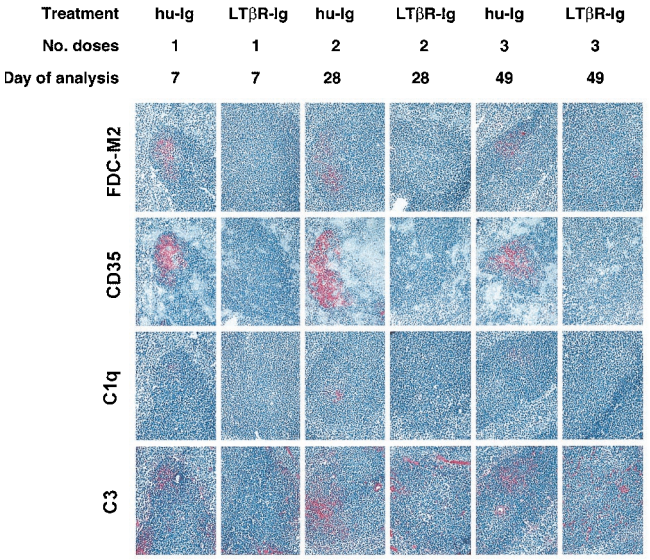

FIG. 3. Effect of multiple hu-Ig or LTβR-Ig treatments on FDC status in spleen. Mice were given a single i.p. injection of 100 μg of LTβR-Ig (or 100 μg of hu-Ig as a control) 3 days before i.p. inoculation with scrapie. After scrapie challenge, mice were then given one or two subsequent doses of 100 μg of LTβR-Ig or 100 μg of hu-Ig at 21-day intervals (days 21 and 42 postinoculation with scrapie). Spleens were taken from two mice from each group 7 days after the last treatment (day 7, day 28, or day 49 after inoculation with scrapie for mice given one, two or three consecutive treatments, respectively). Adjacent frozen sections were stained with FDC-M2 monoclonal antiserum to detect FDCs (top row; red), 8C12 monoclonal antiserum to detect CD35 (second row; red), and monoclonal antisera specific for complement components C1q (third row; red) and C3 (fourth row; red). All sections were counterstained with hematoxylin (blue). After treatment of mice with three consecutive doses of LTβR-Ig, the expression of FDC-M2, CD35, and associated accumulations of complement components C1q and C3 remained undetectable for up to at least 49 days after the first treatment. Original magnification, ×200.

scrapie challenge, mice were then given one or two subsequent 100-μg doses of LTβR-Ig or hu-Ig at a 21-day interval (day 21 and day 42 postinoculation with scrapie). The 21-day treatment interval was chosen to prevent significant FDC recovery between treatments. To monitor the effects of multiple treatments on FDC status, spleens were taken from two mice from each group 7 days after the final treatment. Immunohistochemical analysis demonstrated that treating mice with consecutive doses of LTβR-Ig extended the duration of FDC inactivation (Fig. 3). For example, when mice were given three consecutive doses of LTβR-Ig, the FDC markers FDC-M2 and CD35 remained undetectable for up to at least 49 days after the first treatment (Fig. 3). Likewise, the follicular localization of complement components C1q and C3 was also significantly diminished in the spleen (Fig. 3). Treatment of mice with consecutive doses of hu-Ig (control) had little effect on FDC status (Fig. 3).

In comparison to mice treated with consecutive doses of hu-Ig as a control, treatment with two or three consecutive doses of LTβR-Ig significantly reduced disease susceptibility to i.p. inoculation with a moderate dose of scrapie and extended the survival time (Table 2). However, despite the prolonged effects of treatment on FDC dedifferentiation, consecutive doses of LTβR-Ig had little extra benefit, in terms of susceptibility or survival time, compared to mice given a single LTβR-Ig treatment (Table 2).

Effect of LTβR-Ig treatment on scrapie pathogenesis after oral inoculation. The effects of LTβR-Ig treatment on FDC status are not only confined to the spleen (40). As illustrated in Fig. 4, FDCs in the Peyer's patches and MLNs also temporarily dedifferentiate within 3 days of treatment with LTβR-Ig. Since gut-associated lymphoid tissues have been implicated as potential sites of TSE accumulation and neuroinvasion after oral inoculation, we investigated the effects of temporary FDC inactivation on disease susceptibility after ingestion. Mice were given a single i.p. injection of LTβR-Ig (or hu-Ig as a control) 3 days before or 14 days after oral inoculation with a moderate dose of scrapie (mice were fed individual food pellets doused

TABLE 2. Effect of multiple LTβR-Ig treatments on susceptibility to scrapie[a]

No. of doses[b]	hu-Ig		LTβR-Ig	
	Incidence	Mean incubation period (days) ± SE	Incidence	Mean incubation period (days) ± SE
1	8/8	296 ± 8	5/7	341 ± 22, 2× > 500
2	8/8	305 ± 7	5/8	351 ± 12, 3× > 510[c]
3	9/9	312 ± 13	7/9	369 ± 26, 2× > 510[d]

[a] See Table 1, footnote *a*.
[b] Mice were given a single i.p. injection (100 μg) of LTβR-Ig or hu-Ig (as a control) 3 days before i.p. inoculation with scrapie. Mice were then given injections of LTβR-Ig or hu-Ig (100 μg) at 21-days intervals after scrapie inoculation.
[c] One mouse was killed 414 days after inoculation. No histopathological signs of scrapie were detected in the brain (data not shown).
[d] Two mice were killed 320 and 336 days after inoculation. No histopathological signs of scrapie were detected in their brains (data not shown).

with 50 μl of a 1.0% scrapie brain homogenate). Whereas all mice treated with hu-Ig as a control 3 days before scrapie inoculation succumbed to disease with a mean incubation period of 342 ± 6 days ($n = 8$), treatment with LTβR-Ig had a dramatic affect on disease susceptibility. All mice given LTβR-Ig 3 days before inoculation remained free of the signs of scrapie for at least 518 days postinoculation (Table 3). Characteristic spongiform pathology, PrP^{Sc} accumulation, and reactive astrocytes were detected in the brains of all hu-Ig-treated control animals that developed clinical signs of scrapie (Fig. 5). In contrast, no evidence of spongiform pathology, PrP^{Sc} accumulation, or reactive astrocytes were detected in the brains of any surviving LTβR-Ig-treated mice assayed 518 days postinoculation (Fig. 5).

When mice were treated with LTβR-Ig 14 days after scrapie inoculation, no significant effect on the survival time or disease susceptibility was observed compared to control mice (Table 3). Furthermore, spongiform pathology, reactive astrocytes,

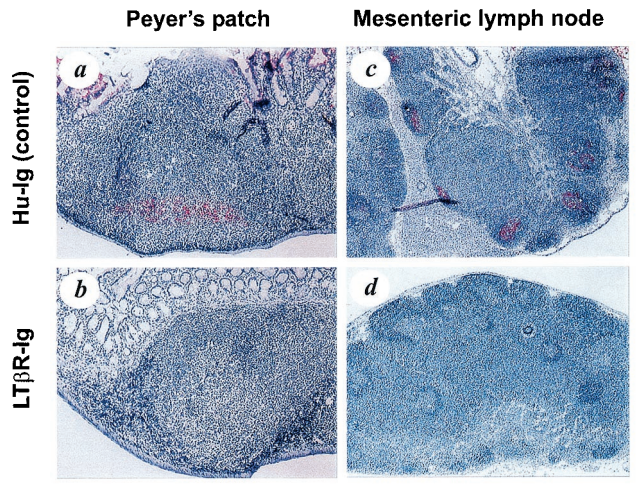

FIG. 4. Effect of LTβR-Ig treatment on FDC status in Peyer's patches and MLNs. Tissues were obtained 3 days postinjection with hu-Ig (control; upper panels) or LTβR-Ig (lower panels), and sections were stained with FDC-M2 monoclonal antiserum (red) to detect FDCs. All sections were counterstained with hematoxylin (blue). Mature FDC-M2-expressing FDCs were undetectable in Peyer's patches and MLNs after treatment with LTβR-Ig. Original magnification, ×200.

TABLE 3. Effect of LTβR-Ig treatment on susceptibility to orally inoculated scrapie[a]

Day of treatment[b]	hu-Ig		LTβR-Ig	
	Incidence	Mean incubation period (days) ± SE	Incidence	Mean incubation period (days) ± SE
−3	8/8	342 ± 6	0/7	7× > 518
+14	8/8	351 ± 12	9/9	356 ± 3

[a] See Table 1, footnote *a*.
[b] Mice were given a single i.p. injection (100 μg) of LTβR-Ig or hu-Ig (as a control) 3 days before (−3) or 14 days after (+14) oral inoculation with scrapie.

and PrP^{Sc} accumulation typical of an infection with the ME7 scrapie strain were detected in the brains of all mice treated with hu-Ig or LTβR-Ig that developed clinical disease (Fig. 5).

Effect of LTβR-Ig treatment on PrP^{Sc} accumulation in Peyer's patches, MLNs, and the spleen. Levels of disease-specific PrP were determined in the Peyer's patches, MLNs, and spleens of mice treated with hu-Ig or LTβR-Ig prior to or shortly after oral inoculation with scrapie. Within 70 days of oral inoculation, strong accumulations of disease-specific PrP were found within the germinal centers of Peyer's patches from control mice treated with hu-Ig 3 days before inoculation (Fig. 6a). The cellular distribution of the PrP was consistent with accumulation in association with FDCs (6, 44). After the early onset of accumulation, high levels of disease-specific PrP were sustained in Peyer's patches of control-treated mice through to the terminal stage of disease (data not shown). However, treatment with LTβR-Ig prior to inoculation blocked the accumulation of disease-specific PrP within Peyer's patches (Fig. 6b). Within the Peyer's patches of these LTβR-Ig-treated mice, only basal levels of staining typical of the cellular form of PrP (PrP^{c}) were detected in the germinal center (6). In contrast, when mice were treated 14 days after inoculation, strong accumulations of disease-specific PrP were found within Peyer's patches from both control and LTβR-Ig-treated mice (Fig. 6c and d, respectively).

A similar pattern of disease-specific PrP distribution was also detected in the MLNs. Within 70 days of inoculation, strong accumulations of PrP^{Sc} were found within the MLNs from control mice treated with hu-Ig 3 days before inoculation (Fig. 6e, lanes 2 and 4). However, treatment with LTβR-Ig prior to inoculation blocked the accumulation of PrP^{Sc} within MLNs assayed 70 days after inoculation (Fig. 6e, lanes 6 and 8). Consistent with the observations made in Peyer's patches (Fig. 6c and 6d), when mice were treated 14 days after inoculation, strong accumulations of PrP^{Sc} were found within MLNs from both control and LTβR-Ig-treated mice when they were analyzed 70 days after inoculation (Fig. 6f).

No PrP^{Sc} was detected in the spleens of any of the control or LTβR-Ig-treated mice assayed 70 days after inoculation (data not shown). However, strong accumulations of PrP^{Sc} were found at the terminal stage of disease in tissues from control mice treated with hu-Ig 3 days before (Fig. 6g, lanes 2 and 4) or 14 days after inoculation (Fig. 6 h, lanes 2 and 4). These data suggest that after uptake from the gut lumen, PrP^{Sc} is propagated from the Peyer's patches to the MLNs via the lymphatics and subsequently distributed to the spleen and other lymphoid tissues, probably via the bloodstream. Consistent with the data

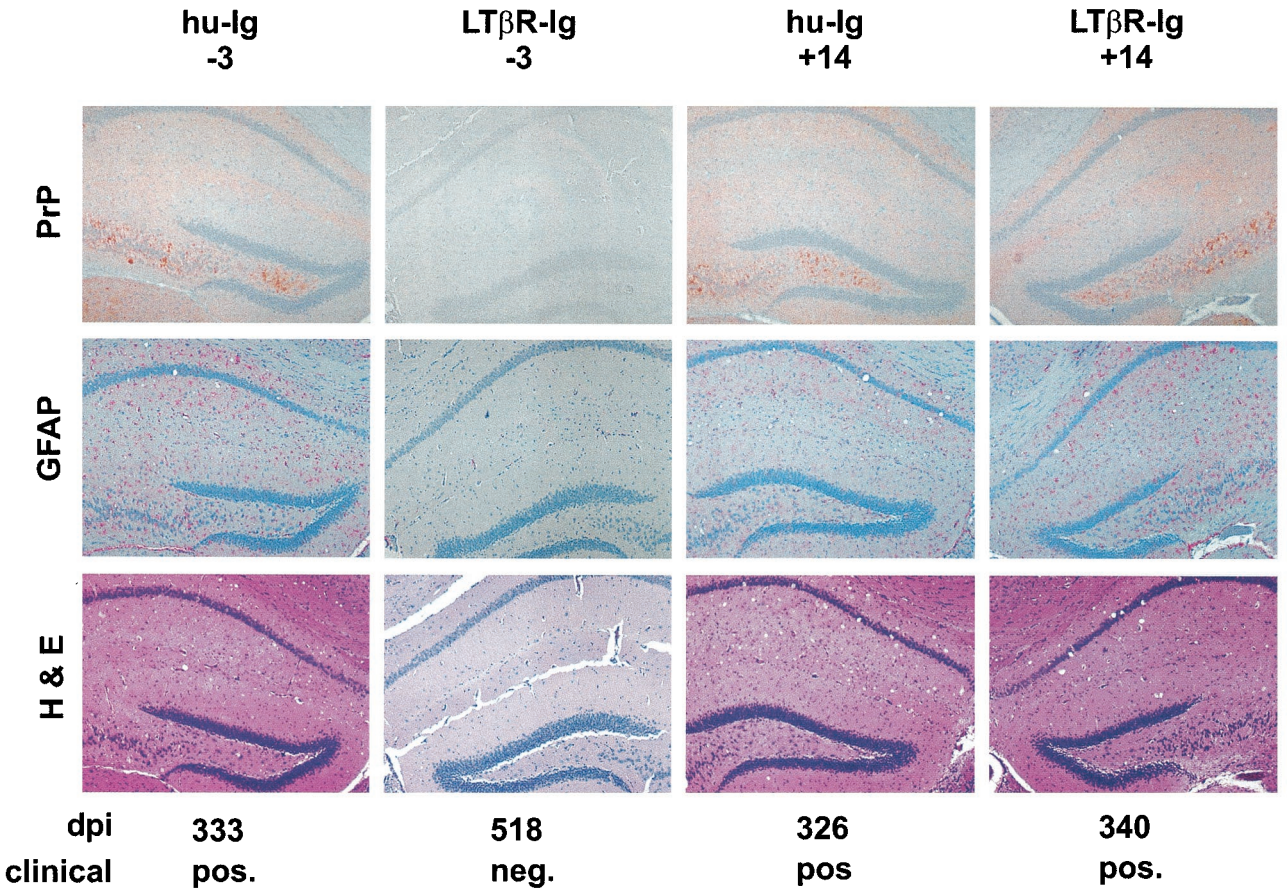

FIG. 5. Immunohistological analysis of brain tissue from mice treated with hu-Ig or LTβR-Ig 3 days before (−3) or 14 days after (+14) oral inoculation with scrapie. Large disease-specific PrP accumulations (upper row; brown), reactive astrocytes expressing high levels of GFAP (middle row; red), and spongiform pathology (H&E; lower row) were detected in the hippocampi of all mice showing clinical signs of scrapie. In contrast, in the brains of mice treated with LTβR-Ig 3 days before inoculation (LTβR-Ig −3), no evidence of PrP accumulation, reactive astrocytes, or spongiform pathology was detected 518 days after inoculation. All sections were counterstained with hematoxylin (blue). dpi, day postinoculation on which the tissues were analyzed; pos., mice that developed clinical signs of scrapie; neg., mice that were free of the clinical signs of scrapie. Original magnification, ×100.

presented above (Fig. 6b and e), PrP^{Sc} accumulation in the spleen was blocked after treatment with LTβR-Ig prior to inoculation since none was detected 518 days after inoculation (Fig. 6g, lanes 6 and 8). Strong accumulations of PrP^{Sc} were found within spleens from mice treated with LTβR-Ig 14 days after inoculation when measured at the terminal stage of disease (Fig. 6 h, lanes 6 and 8).

DISCUSSION

We have shown here that a single treatment of LTβR-Ig before i.p. injection with scrapie significantly extended the survival time and reduced disease susceptibility approximately 100-fold compared to control treated mice. Our studies also demonstrated that treatment prior to peripheral exposure blocked the early accumulation of infectivity and disease-specific PrP^{Sc} within the spleen. These effects coincided with a temporary dedifferentiation of mature FDCs in the spleen for at least 28 days after treatment with LTβR-Ig. Although it was possible to extend the period of FDC dedifferentiation beyond 28 days by treating mice with consecutive doses of LTβR-Ig, prolonged FDC dedifferentiation had little extra effect on susceptibility to a moderate dose of scrapie compared to mice given a single LTβR-Ig treatment. We also demonstrate that mature FDCs are critical for the transmission of scrapie from the gut lumen to the CNS. In the present study, a single treatment with LTβR-Ig prior to oral scrapie inoculation blocked the accumulation of PrP^{Sc} in the Peyer's patches and MLNs and prevented the development of CNS disease. However, treatment with LTβR-Ig 14 days after oral inoculation had no effect on survival time or susceptibility, suggesting infectivity may have spread to the peripheral nervous system by this time.

The effects of LTβR-Ig treatment on FDC status are temporary since the cells recover once the levels of LTβR-Ig in serum fall below a threshold level (22), but in our study there was little evidence of FDC restoration 28 days after injection. Antigens are trapped and retained on the surface of FDCs through interactions between complement components and cellular complement receptors (47, 50). The loss of expression

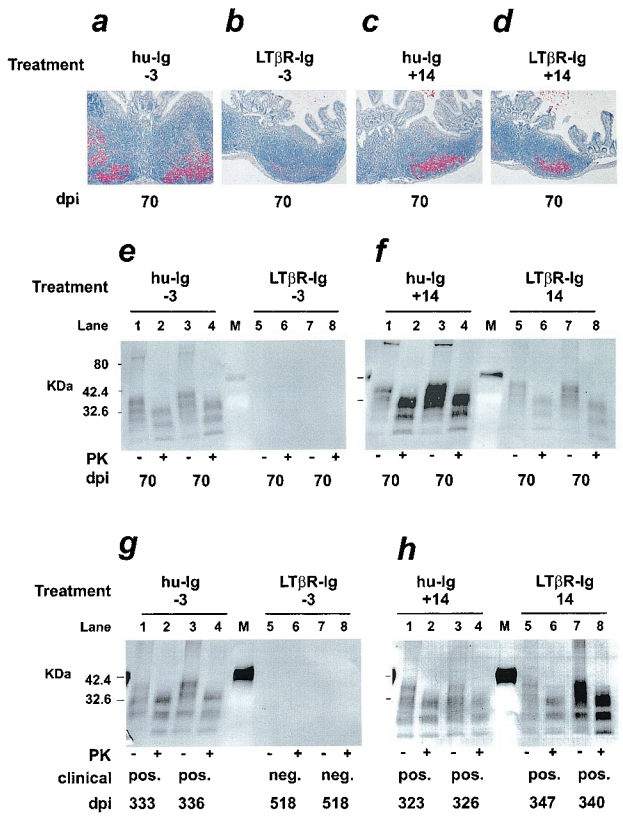

FIG. 6. Treatment with LTβR-Ig prior to oral scrapie inoculation blocks the accumulation of disease-specific PrP in Peyer's patches, MLNs, and spleens. Mice were treated with LTβR-Ig or hu-Ig as a control 3 days before (a, b, e, and g; −3) or 14 days after (c, d, f, and h; +14) oral inoculation with scrapie. Disease-specific PrP accumulations were determined in Peyer's patches (a to d) and MLNs (e and f) assayed 70 days after inoculation and in the spleen (g and h) at the terminal stage of disease. In panels a to d, PrP accumulations in Peyer's patches were detected on paraffin-embedded sections with the PrP-specific polyclonal antiserum 1B3 (PrP, red). Sections were counterstained with hematoxylin (blue). Original magnification, ×100. In panels e to h, immunoblots show the accumulation of detergent-insoluble, relatively proteinase K (PK)-resistant PrP^{Sc} in the MLNs (e and f) and spleens (g and h) of treated mice. Treatment of the tissue in the presence (+) or absence (−) of proteinase K before electrophoresis is indicated. PrP was detected by using the PrP-specific monoclonal antiserum 8H4. Lane M contained molecular mass markers. dpi, day postinoculation on which the tissues were analyzed; pos., mice that developed clinical signs of scrapie; neg., mice that were free of the clinical signs of scrapie.

of complement receptor 1 (CD35) and substantially decreased abundance of complement components C1q and C3 in lymphoid follicles of treated mice indicated that the ability to retain antigens in lymphoid follicles was significantly impaired. Recent studies have identified the antigen recognized by the FDC-specific antiserum FDC-M2 as complement component C4 (55). Thus, the temporary loss of FDC-M2-specific immunostaining after treatment with LTβR-Ig is also consistent with the inability of these cells to trap and retain complement-opsonized antigens. Complement components C1q and C3 and cellular complement receptors have been shown to play an important role in the localization of scrapie infectivity to FDCs (32, 36). Since PrP^{c} expression is also eliminated in lymphoid follicles after treatment with LTβR-Ig (37, 46), these data suggest the capability of FDCs, if present, to acquire and replicate TSE infectivity would be temporarily abolished for at least 28 days.

The fate of the FDCs after LTβR-Ig treatment is not known, but several mechanisms could be responsible either singularly or in combination. First, FDCs could dedifferentiate to an immature state that lacks their antigen-trapping characteristics. Second, in the absence of LTβR stimulation, FDCs may undergo apoptosis. Studies are in progress to determine whether immature FDC processes are detectable at the ultrastructural level or whether there is significant evidence of apoptosis. Finally, since recovering FDCs appear in a more diffuse pattern than the compact networks present in control mice (22), it is possible that a LTβR-dependent chemokine gradient responsible for the localization of FDCs within the germinal center is disturbed, resulting in their dispersal.

After i.p. inoculation of immunocompetent mice with the ME7 scrapie strain, high levels of infectivity are present in the spleen as soon as 35 days postinfection, reaching plateau levels within 70 days postinfection (6, 13, 39). In the present study, when mice were given LTβR-Ig before scrapie challenge, no PrP^{Sc} and only trace levels of infectivity were detected in the spleen at 70 days postinfection, about 40 days after the expected reappearance of mature FDCs. These data are consistent with the requirement for mature FDCs for scrapie replication in lymphoid tissues (6, 17, 31, 37, 39, 46). However, immunoblot analysis of spleens from LTβR-Ig-treated mice that developed neurological disease after protracted incubation periods stained strongly for PrP^{Sc}. The most likely explanation for these observations is that, in the absence of FDCs at the time of scrapie challenge, trace amounts of infectivity from the inoculum persist in the host in a cell or compartment that is not affected by LTβR blockade. Macrophages have been proposed as candidate cells for TSE accumulation in the absence of FDCs (51). The extended survival time in LTβR-treated mice could simply be related to the time required for FDC networks to restore and initiate replication of the inocula. This would delay the subsequent transfer of infectivity via peripheral nerves into the CNS (21). However, LTβR treatment also significantly reduced disease susceptibility, suggesting a substantial portion of the inoculum is destroyed, for example, by macrophages (4, 9). These data are congruent with the suggestion that the action of macrophages on TSE pathogenesis is dose dependent: small doses of infectivity may be easily destroyed by macrophages, whereas higher doses are less easily digested and a fraction is retained.

Although the effects of LTβR-Ig treatment on scrapie pathogenesis are consistent with an absence of mature FDCs at the time of inoculation, our study does not entirely exclude the possibility that other effects of LTβR blockade also make a contribution. For example, blockade of LTβR signaling may have impaired cell trafficking and the transport of infectivity from the site of scrapie challenge to the spleen. Although the direct involvement of migratory bone marrow-derived dendritic cells (DCs [a distinct lineage from FDCs]) in TSE pathogenesis is not known, recent studies suggest that signaling via LTβR by the membrane $LT\alpha_1\beta_2$ heterotrimer is required for their presence in lymphoid tissues (58). However, we have previously demonstrated that LTβR-Ig treatment significantly

extends survival time when given as late as 42 days after scrapie injection (37), making it unlikely that the effects of LTβR blockade on scrapie pathogenesis are due to impaired cell trafficking.

Subsequent experiments were undertaken to determine whether it was possible to further decrease susceptibility to a moderate dose of scrapie by extending the period of FDC dedifferentiation beyond 28 days. Our studies showed that when mice were given three consecutive doses of LTβR-Ig (at 21-day intervals) mature FDCs were undetectable up to at least 49 days after the first treatment. However, despite the prolonged effects of treatment on FDC dedifferentiation, treatment with consecutive doses of LTβR-Ig had little extra protective benefit compared to mice given a single treatment of LTβR-Ig. These data do not exclude the possibility that consecutive treatments with LTβR-Ig would protect mice from inoculation with a 10-fold-lower dose of scrapie (0.1% scrapie brain homogenate) since only two of eight mice developed clinical disease after a single LTβR-Ig treatment (Table 1). Montrasio et al. treated mice with 300 μg of LTβR-Ig prior to scrapie inoculation, followed by seven further doses of 100 μg at weekly intervals (46). Despite the use of a significantly greater amount of LTβR-Ig over a longer time period than that used in the present study, most mice eventually developed clinical disease after i.p. scrapie inoculation (46). The effect of the number or magnitude of the LTβR-Ig doses was not determined in that above study, but our data suggest that a single low dose of LTβR-Ig is almost as effective as two and even three consecutive doses. Prolonged cytokine signaling blockade may cause serious side effects, including increased susceptibility to other infectious microorganisms, increased incidence of malignancies, or induction of autoimmune disease (35). Therefore, if such an approach were ever to have practical use against TSE diseases, treatment would ideally be administered over a short duration to reduce complications from adverse effects.

Although unexpected, these findings (Table 2) are in accordance with studies that indicate that the reduced susceptibility of some immunodeficient mice to TSE challenge can be overcome in a proportion of mice by inoculation with a high dose of infectivity. For example, severe combined immunodeficient (SCID) mice and mice deficient in tumor necrosis factor alpha, LTα, LTβ, or LTβR all lack mature FDCs in lymphoid tissues, but a proportion develop disease after inoculation with a high or moderate dose of scrapie (17, 31, 39, 42, 48, 51). In the permanent absence of FDCs, a study by Prinz et al. (51) suggests that macrophages are plausible candidates for scrapie replication and neuroinvasion from lymphoid organs. However, neuroinvasion after inoculation with a high dose of scrapie possibly also occurs after direct uptake of infectivity by nerve terminals at the site of inoculation or after transport to peripheral nerves by DCs (2).

The suggestion that consumption of BSE-contaminated meat products is the most likely cause of vCJD in humans (7, 25) has focused attention on the gastrointestinal tract as an important portal of TSE entry. Peyer's patches are the primary inductive sites in gut-associated lymphoid tissues that actively acquire antigens from the lumen of the intestine. After intragastric or oral inoculation of rodents with scrapie, infectivity and PrP^{Sc} accumulate first in Peyer's patches, gut-associated lymphoid tissues, and ganglia of the enteric nervous system long before their detection in the CNS (3, 30, 41, 45). Natural sheep scrapie may also be acquired orally as PrP^{Sc} is detected in Peyer's patches and gut-associated lymphoid tissues (1) prior to detection within the CNS (57). Within the gut-associated lymphoid tissues of orally inoculated rodents (3) or sheep (1, 23) with scrapie and mule deer inoculated with CWD (54), disease-specific PrP accumulations occur in association with FDCs. In the present study, temporary FDC inactivation prior to oral inoculation prevented the development of clinical disease and blocked the accumulation of PrP^{Sc} in Peyer's patches and MLNs. These data demonstrate that mature FDCs are critical for the transmission of scrapie from the gut lumen to the CNS and exclude the possibility of direct uptake of infectivity from the gut lumen into PrP^{c}-expressing enteric nerves (53).

We have previously shown that temporary FDC inactivation significantly extends survival time when treatment with LTβR-Ig is delayed for up to at least 42 days after i.p. scrapie inoculation (37). Surprisingly, in the present study treatment with LTβR 14 days after oral inoculation with scrapie did not significantly affect survival time or disease susceptibility. The levels of infectivity responsible for natural TSE transmissions are not known but are likely to be much lower than the moderate dose used in the present study. Further studies will determine whether treatment reduces susceptibility when given 14 days (or later) after oral inoculation with lower doses of scrapie. In the Peyer's patches and MLNs of immunocompetent mice, replication of scrapie on FDCs is likely to have already begun within 14 days of inoculation. Treatment with LTβR-Ig has no affect on disease pathogenesis once infection is established within the CNS (37). The lack of any observable effect of LTβR-Ig treatment on disease pathogenesis when given 14 days after oral inoculation is therefore consistent with the hypothesis that neuroinvasion has already occurred. Recent studies have demonstrated that PrP^{Sc} accumulates within enteric ganglia soon after oral inoculation (3, 45). In the Peyer's patch, FDC networks are situated distal from the gut lumen and lie in close association with nerve fibres that run along the gut wall (3). Therefore, it is plausible to suggest that after accumulation and/or replication upon FDCs, neuroinvasion occurs rapidly and most likely via the enteric nerves in the gut wall or Peyer's patch adjacent to FDCs (3, 45). Our studies do not exclude the possibility that infection also spreads to the peripheral nervous system from the MLNs at a similar time point, but the lack of detectable PrP^{Sc} in the spleen 70 days after inoculation suggests the spleen does not play an early role. Thus, these data illustrate that LTβR-Ig may be used as a tool in subsequent experiments to determine precisely the onset of neuroinvasion after peripheral inoculation.

Treatments that inactivate FDCs may have therapeutic application in some other pathological conditions. For example, FDC inactivation may reduce the severity of some autoimmune diseases. Also, FDC-associated human immunodeficiency virus particles comprise a major viral reservoir in infected patients (8), and removal may improve the efficacy of antiviral therapies. Moreover, the elimination of FDCs may reduce the survival of some lymph node-resident lymphomas that require FDCs for their survival (22, 29). Current evidence suggests that the human TSE disease, vCJD, shares a similar

requirement for FDCs in its pathogenesis, since PrP^{Sc} is associated with FDCs in lymphoid tissues from infected patients (24) and has been detected in the appendix prior to the onset of clinical disease (26, 27). Thus, manipulation of FDCs may offer a potential approach for early intervention in peripherally acquired TSEs. However, treatments that specifically interfere with the maturation of FDCs will only be effective during the time interval between exposure to infection and spread of disease to the peripheral nervous system. Although little is known about the precise timing of these events, comparisons of the effects of LTβR-Ig treatment on scrapie pathogenesis when inoculated via the peritoneal cavity (37) or gastrointestinal tract (current study) suggest that these effects may vary widely depending on the route of exposure. Therefore, LTβR-Ig may be used as a tool in future experiments to precisely determine the duration of FDC involvement in TSE pathogenesis.

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